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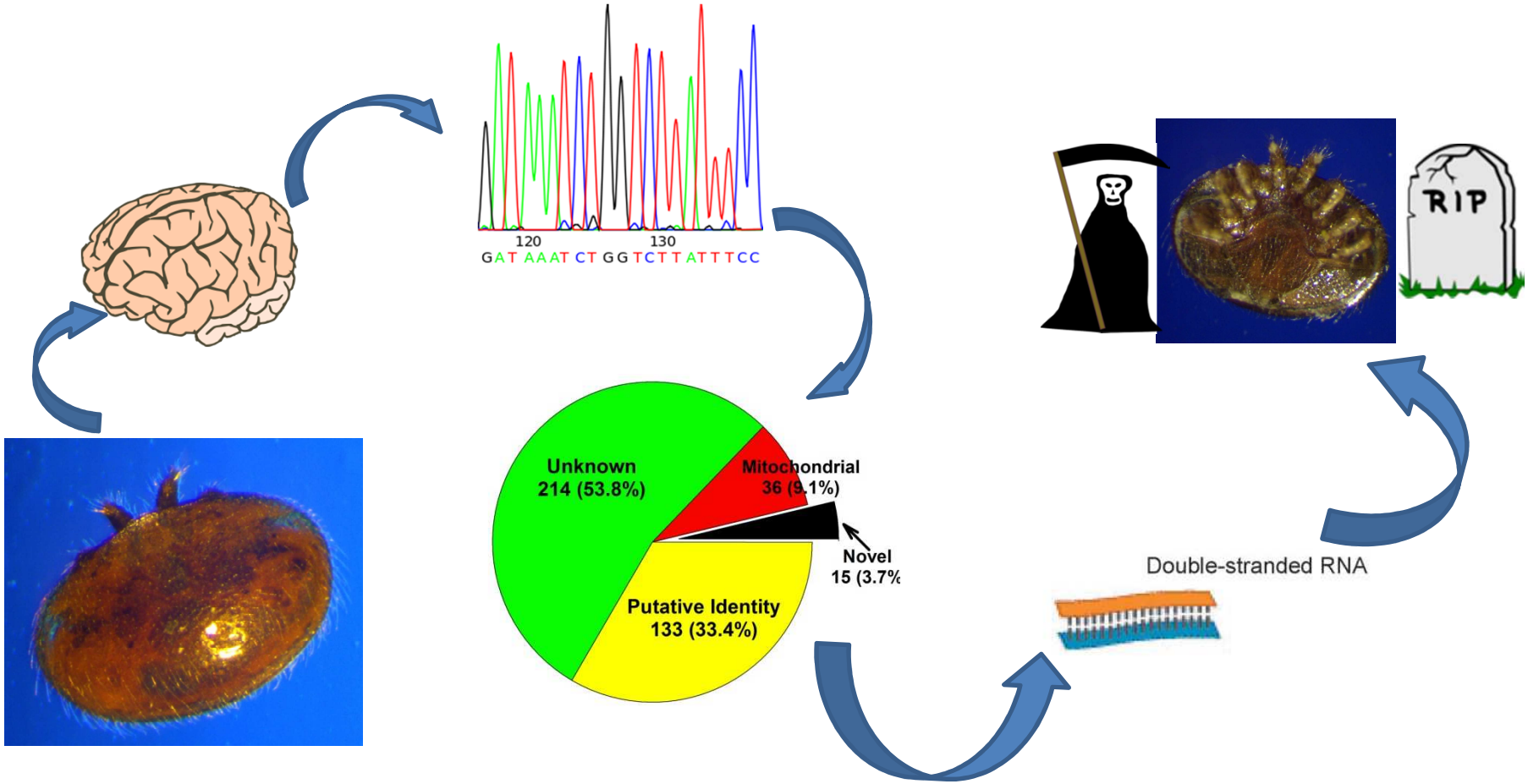
120
130
GAT AAAT CT GG TCTT ATTTCC
Unknown
214 (53.8%)
Mitochondrial
36 (9.1%)
Novel
15 (3.7%
Putative Identity
133 (33.4%)
Double-stranded RNA
RIP

Transcriptome analysis of the synganglion from the honey bee mite, *Varroa destructor* and RNAi knockdown of neural peptide targets.

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Abstract:

Varroa mites (*Varroa destructor*) and the viruses that they transmit are one of the major contributing factors to the global honey bee crisis. Gene products within the nervous system are the targets of all the insecticides currently used to control *Varroa* but there is a paucity of transcriptomic data available for *Varroa* neural tissues. A cDNA library from the synganglia ("brains") of adult female *Varroa* was constructed and 600 ESTs sequenced and analysed revealing several current and potential druggable targets. Contigs coding for the deformed wing virus (DWV) variants *Varroa destructor* virus-1 (VDV-1) and the recombinant (VDV-1_{DVD}) were present in the synganglion library. Negative-sense RNA-specific PCR indicated that VDV-1 replicates in the *Varroa* synganglion and all other tissues tested, but we could not detect DWV replicating in any *Varroa* tissue. Two neuropeptides were identified in the synganlion EST library: a B-type allatostatin and a member of the crustacean hyperglycaemic hormone (CHH) superfamily. Knockdown of the allatostatin or the CHH-like gene by double-stranded RNA-interference (dsRNAi) resulted in 85% and 55% mortality, respectively, of *Varroa*. Here, we present the first transcriptomic survey in *Varroa* and demonstrate that neural genes can be targeted by dsRNAi either for genetic validation of putative targets during drug discovery programmes or as a potential control measure in itself.

Keywords:

Varroa destructor, *Apis mellifera*, synganglion, deformed wing virus, allatostatin, crustacean hyperglycaemic hormone, gene knockdown

1. Introduction

The economic contribution of insect pollination to crop production is significant (Gallai et al., 2009) and managed honey bees provide about half of this ecosystem service to all insect-pollinated crops on Earth (Kleijn et al., 2015). The critical importance of honey bees (*Apis mellifera*) to global food production, and the central role of *Varroa destructor* to *A. mellifera* colony losses, elevate *Varroa* to arguably the most serious parasite of any livestock species worldwide. *V. destructor* is an ectoparasitic mite of honey bees that is now endemic in all countries where husbandry of honey bees occurs, except Australia (vanEngelsdorp et al., 2009,Martin et al., 2013,vanEngelsdorp and Meixner, 2010). *V. destructor* are vectors of at least 18 different honey bee viruses, including highly pathogenic strains of deformed wing virus (DWV) (Di Prisco et al., 2011,Wang et al., 2013,Genersch and Aubert, 2010). The recent spread of *Varroa* coupled with its ability to act as a vector for DWV has resulted in a synergistic effect that has been heavily implicated in a global decline in honey bee health (vanEngelsdorp et al., 2009,Martin et al., 2012). *Varroa* and associated pathogens have led to higher than average winter losses in many regions (Highfield et al., 2009), resulting in increased effort and money spent on combating infestations and replacing stock. Typically, hives infested with *Varroa* are unlikely to survive without intervention for more than 1-3 years (Martin, 1998). Integrated pest management strategies including drone brood culling, mite population monitoring and the use of various organic acids during broodless periods can significantly reduce *Varroa* burden (Rosenkranz et al., 2010). Breeding *Varroa* resistant or tolerant *A. mellifera* offers great potential, but, is not yet a reality (Correa-Marques et al., 2002). The control of *Varroa* still remains largely based on chemical acaricides. There is now widespread and increasing resistance to tau-fluvalinate, flumethrin, coumaphos and amitraz (Rodriguez-Dehaibes et al., 2011), leaving many areas with no effective control measures against *Varroa* during much of the beekeeping season. The current lack of alternative control methods may undermine the future of sustainable apiculture globally (Dietemann et al., 2012) and, hence, general food security.

In common with other pesticides, the majority of acaricides target vital pathways and channels within the nervous system (Lees et al., 2010) and it is highly probable that the development of new acaricides will also target this tissue. Sequencing of the *V. destructor* genome and tissue-specific transcriptome libraries, along with reverse genetic approaches such as gene-knockdown by RNA interference (RNAi) can help to identify physiologically important pathways and inform selection of viable targets for therapeutic control. Given the

importance of *Varroa* to global food production, there are rather limited genetic resources other than an initial genomic survey (Cornman et al., 2010). Presently, there is an absence of any neural-tissue specific *Varroa* sequences in public databases other than the voltage-gated sodium channel (Wang et al., 2003), the target of pyrethroids.

RNAi is used widely as a tool for investigating gene function in many taxa, including arachnids (Karim et al., 2008,Browning and Karim, 2013), and has been employed to investigate the physiology as well as inform acaracide development in numerous tick species. RNAi is effective in the two-spotted spider mite *T. urtica* (Khila and Grbic, 2007) and recently was demonstrated to be effective in *V. destructor* directly, through injection and immersion of dsRNA (Campbell et al., 2010) or via feeding dsRNA to honey bees on which *Varroa* are present (Garbian et al., 2012). To date, RNAi in *Varroa* has been limited to targeting multi-tissue housekeeping transcripts, such as V-ATPase subunits and others involved with cellular homeostasis or detoxifying enzymes ubiquitously expressed in all tissues. RNAi has huge potential for applied entomology (Scott et al., 2013,Price and Gatehouse, 2008). Neural targets for the development of pesticides can be tested by high throughput RNAi gene-knockdown (Lees and Bowman, 2007) and it has become increasingly apparent that RNAi has the potential itself to manage pests and parasites by targetting transcripts that are vital to pest physiology by suppressing vital genes to reduce reproductive capacity and increase mortality (Scott et al., 2013). It is reasonable to postulate that the synganglion is the site of numerous genes highly critical to the *Varroa*'s survival, but the sequence of such genes is not currently available.

In the current study, we constructed and sequenced an EST library from *Varroa* synganglia to identify neural genes that may be potential targets for *Varroa* control. We then investigated the potential for dsRNA to silence two neurohormone genes identified within the *Varroa* synganglion library. Both targets were inhibited by dsRNA and displayed individual phenotypic responses to RNAi including increased mortality. This work demonstrates dsRNA can gain access to the synganglion of *Varroa* enabling further investigation into mite neurophysiology and inform future methods of control. The lethality of neural peptide gene knockdown also heralds the possibility of *Varroa* control by RNAi of neural tissue –specific targets.

2. Materials and methods

2.1. EST library construction

Varroa destructor (adult female) mites were collected from capped brood cells frames from *Apis mellifera* hives from York, England that had purposefully been left untreated for *Varroa* control. Prior to harvesting mites, the bee frames were maintained at 27ºC and 80% relative humidity environment with a 15.5 h : 8.5 h light:dark regime. Approximately 50 synganglia were dissected from mites in phosphate buffered saline (PBS) before being washed in sterile ice-cold PBS and pooled together in a 1.5 ml eppendorf microfuge tube containing 200 µl RNA-*later* (Sigma, Poole, UK). De-synganglionized mite bodies as well as Malpighian tubules, gut and whole mites were also retained. Prior to RNA extraction, an additional 450 µl dissection buffer was added to sample tubes and centrifuged at 20,000 RCF for 15 min at 4°C. Supernatant was removed and the synganglion washed with fresh PBS before a final centrifuge again at 20,000 RCF for 15 min at 4°C. Supernatant was again removed and 600 µl ZR extraction buffer added to each tissue sample. Total RNA was extracted using a mini-RNA isolation II Kit (Zymo Research, Orange, California, USA), as per manufacturer's instructions and eluted in 50 µl water. RNA was co-precipitated with 1.5 µl glycogen blue (Life Technologies, Paisley UK) and 5 µl 3M sodium acetate in 95% ethanol and resuspended in 5 µl of DEPC-treated water.

First strand cDNA synthesis was completed using 3.5 µl (0.5 µg) of total RNA. The construction of cDNA libraries was done using the SMART cDNA library construction kit (Clontech, St-Germain-en-Laye, France) according to the protocol provided by manufacturer, with some modifications. To determine optimal number of cycles, two identical amplification reactions were prepared. After the 10th amplification cycle the first reaction was stored on the ice, while the second one was used for the PCR cycle number optimization by removing 3 µl samples from the reaction tube every two cycles until cycle number 20. Samples were checked by visualization on a 1.1% agarose gel. The optimal number of cycles with visible and equally represented products, in this case 20 cycles, was used for primary amplification. cDNA was proteinase K treated extracted using phenol:chloroform and resuspension in water. After *Sfi*I digestion and size fractionation with a Chroma Spin-400 column, fractions were checked using agarose gel and pooled into large or medium libraries. Pooled cDNA was ethanol precipitated and eluted in 4 µl of water prior to 3 µl from each fraction being ligated into the λTripleEx2 vector and packed into phage using the Gigapack III Gold Packaging extract (Stratagene). Each un-amplified library was mixed with *E.coli* XL1 blue cells and top

agar supplemented with X-gal and IPTG before being plated onto LB $MgSO_4$ agar plates in serial dilutions of neat, 1:10, 1:100 and 1:1000.

In total, 600 randomly selected recombinant plaques (white) were picked as agar plugs into 96-well plates, each well containing 100 µl of SM buffer (0.58% NaCl, 0.2% $MgSO_4 \cdot H_2O$, 0.05M Tris-HCl, pH 7.5, 0.02% gelatin). Four plates (384 clones) were picked from the large fraction library, two plates (192 clones) from the medium fraction library and an additional 24 clones from the large fraction library for initial quality control. PCR with vector-specific primers was carried out using SM buffer / picked plaques as templates. PCR was carried out in 96-well plates containing 25 µl 2x Biomix (Bioline), 5 µl template, 1 µl (10 ng/µl) each of PT2F1 (5'- AAGTACTCTAGCAATTGTGAGC-3') and PT2R1 (5'- CTCTTCGCTATTACGCCAGCTG- 3') and 18 µl water to give a 50 µl final reaction volume. Cycling conditions were 94°C for 15 min followed by 33 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 1 min 20s. PCR products were sent to GATC (Konstanz, Germany) for PCR reaction clean up and sequenced using primer PT2F3 (5' – CTCGGGAAGCGCGCCATTGT- 3'). PT2F3 is upstream from inserted cDNA and downstream from PT2F1 primer used in initial PCR reaction.

2.2. Bioinformatics

Expressed sequence tags (ESTs) were trimmed of primer and vector sequences, clustered and checked for sequence quality using Lasergene Seqman (Lasergene v8.03, DNAstar, Madison, USA). BLASTn, BLASTx and tBLASTx programmes were used within the programme BLAST2GO to compare the EST nucleotide sequences with the nonredundant (NR) databases of the NCBI and to the Gene Ontology (GO) database (www.blast2go.org). BLASTn against the *Varroa* WGS genome was carried out using the NCBI BLAST servers. Following analysis of results, transcripts were initially classified as novel sequences, putative identity or unknown function. Transcripts with a putative identity were further divided into functional categories by analysing GO identity and homology to known genes.

Neurohormone peptides were identified by signal peptide sequence, pro-hormone processing sites and length (<300 amino acids). Signal peptide analysis was carried out using SignalP (http://www.cbs.dtu.dk/services/SignalP/) and Signal-3L (http://www.csbio.sjtu.edu.cn/bioinf/Signal-3L/) using both the neural networks and Hidden Markov Model algorithms. Pro-hormone cleavage sites were predicted based on work by

Veenstra (Veenstra, 2000), and Neuropred (http://neuroproteomics.scs.illinois.edu/neuropred.html). Predicted transmembrane topology of the neurohormones was predicted with TMHMM (Krogh et al., 2001) at http://www.cbs.dtu.dk/services/TMHMM/.

The neurohormones crustacean hyperglycaemic hormone (CHH) and allatostatin (AST) sequences were primarily aligned in GUIDANCE using the progressive algorithms PRANK and MAAFT (http://guidance.tau.ac.il/) with confidence scores assigned for each residue, column, and sequence. Unreliable columns and sequences were removed from the initial alignment in preparation for downstream analyses. MEGA 6.3 (Tamura et al., 2013) was used to align sequences by MUSCLE. Phylogenetic trees were constructed by Maximum likelihood using the LG (+G+I) model of analysis and bootstrapped 1000 times.

2.3. Virus identity, distribution and replication

Three sequences with homology to *Varroa destructor* virus-1 (VDV-1) were found in the EST library as determined by BLASTn. Alignment comparisons were carried out with MEGA(6.3) using genome data from known VDV-1 and DWV strains (Moore et al., 2011,de Miranda et al., 2013). Pairwise identity comparison was determined by CLUSTAL-omega.

To determine the variety of DWV strains present in the synganglia of *Varroa*, as well as tissue specificity, a nested-PCR approach was taken. Generic primers spanning the major recombination junction between DWV and VDV-1 were used in the first round PCR (Moore et al., 2011) using synganglia EST library material as well as cDNA constructed from gut, Malpighian tubules and de-synganglionized mite carcass. Nested-PCR was then carried out with variant specific primers and 1 µl of first-round product. Products were visualized by gel electrophoresis.

Virus replication was assessed by the specific detection of negative strand viral RNA using strand-specific Reverse Transcriptase-PCR (Yue and Genersch, 2005). 0.5 µg of mite RNA extracted from synganglia, gut, Malpighian tubules and de-synganglionized mite carcass (see above) was reverse transcribed with thermostable Superscript III (Life Technologies, Paisley, UK) and tagged with first-strand primers specific for either DWV or the VDV variant of the virus. Reverse transcription was carried out at 55°C for 1 hour and treated with RNAse-H to remove the possibility of false positive detection during subsequent PCR. A template-free cDNA reaction was carried out as a control. Tissue specific virus-primed cDNA was

subjected to qPCR by SybrGreen detection on an Opticon II platform PCR cycling conditions were as follows: 1 cycle of 10 min at 95°C, followed by 35 cycles of 20s at 94°C, 20s at 55°C and 20s at 72°C. Control reactions with primer and template free reaction mixture were included. To confirm product specificity, melting point analysis was performed at the end of the qPCR run and the products analysed by gel electrophoresis.

2.4. Spatial distribution of neural peptides

To determine if CHH and AST transcripts are restricted to the synganglionic tissue of *Varroa*, RNA extracted from whole mites, de-synganglionized mites, gut tissue and Malpghian tubules was reverse transcribed. After isolation, 0.5 µg total RNA was DNase treated with 1 µl (2U) RQ1-DNase (Promega, Southampton, UK) and 1 µl RQ1 buffer and incubated at 37°C for 30 min. DNase-treated total RNA was incubated at 70°C with 0.5 µg of oligo d(T)15 (Promega) in a total volume of 10 µl for 5 min. Material was snap-chilled on ice for 5 min prior to the addition of 5 µl 5×RT buffer, 1 µl dNTPs (25 mM each), 0.5 µl Bioscript-reverse transcriptase and DEPC water to 25 µl. The reaction was incubated at 42°C for 1 hour. PCR was carried out with CHH, AST and *Varroa* actin specific primers using the following cycling conditions: 1 cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 53°C and 45 s at 72°C. Products were resolved on an agarose gel and visualised by UV light.

2.5. Preparation of dsRNA

*Vd*CHH and *Vd*AST dsRNA was prepared with BLOCK-iT RNAi TOPO transcription kits (Invitrogen, Paisley, UK), according to the manufacturer's instructions. LacZ-dsRNA was prepared and used as a negative control. Briefly, PCR was carried out using adult female *V. destructor* cDNA in conjunction with *Vd*CHH specific primers (*Vd*CHH F1/R1), *Vd*AST specific primers (VdAST F1/R1) or with control LacZ-plasmid and LacZ specific primers (LacZ-F2, ACCAGAAGCGGTGCCGGAAA and LacZ-R2, CCACAGCGGTGGTTCGGAT). PCR cycling conditions were as described above followed by a final extension time of 15 min at 72°C. Products were resolved on an agarose gel, excised and purified using a Qiagen gel extraction kit (Qiagen, Crawley, UK). TOPO-T7 linker was ligated to *Vd*AST, *Vd*CHH and LacZ reactions before a secondary PCR was carried out to produce sense and antisense templates. T7-RNA polymerase was used in transcription reactions with target templates to generate sense and antisense RNA. Single RNA strands were annealed and the resultant dsRNA purified and quantified by a ND-1000

Nanodrop Spectrophotometer (Labtech Inc., East Sussex, UK). dsRNA was ethanol precipitated and resuspended in DEPC-treated water to a working concentration of 2.5 μg/μl and stored at -80°C.

2.6. Administration of dsRNA by injection and immersion

Adult female *V. destructor* were removed from capped brood cells along with associated bee larvae. Microinjections were carried out according to methods previously described (Campbell et al., 2010). Briefly, mites were placed on double-sided tape ventral side up, and injected with 20 nl (2.5 μg/μl) of either *Vd*CHH-dsRNA, VdAST-dsRNA or LacZ-dsRNA in the soft tissue either proximal to the anal region or postcoxal plate using pulled glass capillary needles in conjunction with a Harvard micro-injector system. Mites were placed in petri dishes containing 1 bee larvae per 4 mites. Dead or unhealthy looking mites, presumably due to the injection trauma, were discarded after 1 hour. To assess penetration of neural tissue by the immersion method (Campbell et al., 2010,Campbell et al., 2010), adult mites were removed from capped brood cells and placed in 500 μl microfuge tubes containing 20 μl *Vd*CHH-dsRNA, VdAST-dsRNA or LacZ-dsRNA (2.5 μg/μl) in 0.9% NaCl. Mites were soaked at 4ºC overnight, then removed, dried and placed in Petri dishes. Mites were fed on similar aged developing bee larvae (replaced every 24h) and maintained at 30°C and 85% RH.

2.7. Validation of RNAi

RNA was extracted from individual *Varroa* 48 h post-treatment. In addition, persistence of the RNAi effect was measured in injected mites by harvesting at 18, 24, 48 and 72 h. Total RNA was extracted from mites using ZR Insect RNA MicroPrep (Zymo Research, CA, USA), DNase-treated and reverse transcribed. PCR was carried out using either *Vd*CHH-dsRNA, *Vd*AST-dsRNA or LacZ-dsRNA treated sample cDNA in conjunction with primers specific for actin or *Vd*CHH / *Vd*AST using primers and cycling conditions, as described above. Products were visualized on an agarose gel normalized to actin loading. To assess persistence and approximate % knockdown, ImageJ software was used to carry out semi-quantitative densitometric analysis on gel images.

2.8. Phenotypic responses to RNAi

To assess mortality induced by neurohormone gene knockdown, adult female *Varroa* were removed from capped brood cells, treated with dsRNA by the immersion method described

above and maintained on developing bee larvae at 30°C and 85% RH. *Varroa* were monitored every 8 hours for up to 5 days and dead mites removed and counted. "Self righting" behaviour was assessed during inspections to determine morbidity and other non-typical behaviour was noted.

3. Results

3.1. Synganglion EST database summary

A *V. destructor* EST library derived from the synganglia of 50 mites' RNA was constructed. The large fragment library consisted of 6.23 $x10^6$ colony forming units (cfu)/ml and the medium fragment library consisted of 1.07 $x10^7$ cfu/ml with recombination of 94.3 and 96.3%, respectively. A total of 600 plaques were picked from plates from the large and medium fractionated libraries and sequenced. 555 (92.5%) ESTs were of sufficient length (>75 bp) and quality for further analysis and assembled into 398 clusters, with a mean length of 484 bp, ranging from 92 – 899 bp. Of these clusters, 11 had more than one associated EST, with a mean of 15.2 ESTs / contig. The top three best coverage contigs were *V. destructor* mitochondrial genes; 16S ribosomal protein, 12S ribosomal protein and cytochrome oxidase containing 80, 28 and 10 ESTs, respectively. The remaining ESTs consisted of 387 singletons (Supplementary Table 1).

All clusters were initially compared by BLASTn and BLASTx to the *Varroa destructor* WGS (PRJNA33465) and mitochondrial (AJ493124.2) genomic databases. 381 clusters (95.7%) were homologous to database sequences. The remaining 17 clusters were BLASTed against other nr and protein databases using BLASTn and tBLASTx, respectively. A single cluster containing 3 ESTs from this subset was homologous to a region of *Varroa destructor* virus-1 (VDV-1), a pathogenic variant of DWV. Viral contigs were located near the 3'end of the VDV-1 genome in a region coding for RNA-dependant RNA polymerase (RdRp). No classic DWV sequences as normally present in bees were isolated from the synganglia EST.

Clusters were BLASTed against NCBI nucleotide and protein collection (nr/nt) and assigned into categories (Fig. 1A). Of the 398 clusters analysed, 3.7% were novel and 33.4% had high homology to annotated sequences (Supplementary Table 1). The remaining clusters were homologous to existing *Varroa* genomic databases with no annotation or known identity. The 3.7% of sequences with no homology to the *Varroa* genome were BLASTed against other databases but no identity was determined. It may be that future versions of the *Varroa*

genome or on-going transcriptome studies will help with the identification of these contigs. Clusters with putative shared identity with existing sequences in the nr/nt database were compared with the GO database using BLAST2GO and assigned functional classification (Fig. 1B). The largest functional sets were for clusters associated with metabolism (24.7%) as well as cell growth, division or RNA transcription (20.3%). The potentially druggable target categories of transmembrane receptors and neurohormones accounted for 6.5% of synganglion library clusters. Sequence data and initial homology searches for all 398 clusters are given in Supplementary Table 1.

3.2. Virus identity, distribution and replication

Three contigs (L4C04, MIC05 and L1A04) coded for DWV variants. Contigs M1C05 and L1A04 were identical to each other and were treated in the analysis as such. L1A01 and L4A04, sequenced from the EST synganglia database both had >99% identity with VDV-1 (Genbank AY251269.2) but lower identity to DWV (NC_004830.2), 91% and 84%, respectively (Fig. 2a). To determine the DWV strain diversity in the synganglia of *V. destructor* we carried out strain-specific PCR across the identified recombination point. Nested PCR resulted in bands of the expected size for both VDV-1 as well as the recombinant VDV-1_{DVD} but DWV and recombinant VDV-1_{VVD} were absent (Fig. 2b).

To determine if VDV-1 is replicating in *Varroa* synganglia, we assayed for negative-sense RNA strands as during replication the positive-sense RNA of VDV-1 is transcribed to a full-length copy of the negative-sense RNA strand genome. Reverse transcription was carried out on RNA extracted from *Varroa destructor* tissues using VDV-1 or DWV specific primers and thermostable transcriptase. qPCR of VDV-1- or DWV–primed cDNA detected the presence of negative-strand RNA from VDV-1 in all tissues assayed as determined by gel electrophoresis (Fig. 2c) and product melting point analysis (data not shown). No negative-strand DWV was detected in any tissue assayed

3.3. Neuropeptide sequence analysis

3.3.1. CHH/GIH/MIH/CHH contig

A 705 bp contig found in the EST library (L1C01) was identified by BLAST to have high homology to crustacean hyperglycaemic hormone (CHH) from *Homarus americanus* (2105187B) and *Callinectes sapidus* (AAS45136) and to ion transport peptide (ITP) from the

tick *Dermacentor variablis* (ACC99599.1). This sequence was identical to a region of the *Varroa* genome WGS (VDK00079049) and had high homology (88%) to a sequence found in the Western predatory mite, *Metaseiulus occidentalis*, genome (XM_003741077). No hits were returned when the coding region was BLASTed against the spider mite *Tetranychus urticae* WGS database.

In silico analysis of L1C01 contig demonstrated a coding region of 134 amino acids with a crustacean hyperglycemic hormone/molt-inhibiting hormone/gonad-inhibiting hormone (CHH/MIH/GIH) motif (Pfam PF011147) at positions 54 to 129. SignalP revealed a 28 amino acid signal peptide cleaved at position T28/Q29. The *M. occidentalis* sequence also contained the CHH/MIH/GIH motif and a signal peptide cleavage site at position A24/F25. Both sequences contain 6 canonical cysteine residues conserved in the CHH superfamily (Fig. 3c).

DELTA-BLASTp interrogation of the nr protein database with the L1C01 sequence returned multiple hits to members of the CHH-superfamily with six conserved cysteine residues identical across all results. Interestingly, most homology values are relatively low with maximum identities between 26 and 35% for sequences with significant expect values of 9e-05 and below. The top hit was an uncharacterized protein from the western predatory mite, *Metaseilulus occidentalis.* The top annotated hits are CHH sequences from various marine crustaceans whereas the highest ITP match was from the tick, *Dermacentor variablis*. Top BLASTp hits and selected others mined from the *T. urtica* genome and tick databases were subjected to GUIDANCE analysis, aligned using MEGA6.3 and maximum-likelihood phylogenetic trees were constructed. Both *Varroa* and *M. occidentalis* sequences shared a branch along with other Acari sequences separated from both CHH, MIH/GIH and ITPs (Fig. 3). Although phylogenetic analysis determined the L1C01 contig resides within a distinct acarine clade, no inference of function was revealed and bootstrap values were relatively low between all clades.

3.3.2. Allatostatin contig

A 708 bp sequence found in the synganglia EST library (M2D11) was identified by BLAST to have homology (42%) to B-type allatostatin (AST) from *Aedes aegypti* (XP_001655873.1). This sequence was identical to a region of the *Varroa* genome WGS (VDK00000794) and had high homology (62%) to a sequence found in the Western

predatory mite, *Metaseiulus occidentalis*, genome (XP_003741714). *Varroa* AST belongs to the B-type AST as it contains the conserved peptide sequence motif W(X)6W and a weak signal peptide cleavage point between position A31 and V32 (Fig. 4a). The *M. occidentalis* AST also contains W(X)6W motifs and signal peptide cleavage point between positions A21 and K22. Similar to other arthropod B-type ASTs, *Varroa* AST has multiple lysine and arginine cleavage sites at positions 69/70, 87/88, 101/102, 112/113 and 127/128 that would result in three mature peptides 16, 12 and 13aa in length each containing the W(X)6W motif (Fig. 4b). Though three acarine B-type ASTs (*Varroa*, *M. occidentalis* and *I. scapularis*) contain three mature peptides, *T. urtica* contains six.

3.4. Neural peptide transcript distribution

RT-PCR demonstrated that AST is confined to the synganglion with no detection in Malpighian tubules, gut or de-synganglionized mite carcass. In contrast, though CHH was abundantly detected in the synganglion, it was lowly expressed in gut and the de-synganglionized carcass and very weakly present in Malpighian tubules (Fig. 5).

3.5. RNAi knockdown of neuropeptides

Successful knockdown for both AST and CHH was initially performed by intrahaemocoelic injection of the corresponding dsRNAi (data not shown). All subsequent knockdown experiments and presented data administered the dsRNA by immersion which allows more *Varroa* to be treated and decreases mortality from the injection trauma. *Varroa* treated with dsRNA-AST showed a decrease in AST mRNA levels within 24 hr and decreasing further by 85% knockdown ($P < 0.001$) of target gene after 48h compared to the control *Varroa* group treated with dsRNA- LacZ (Fig. 6a). Mites treated with dsRNA-AST exhibited significantly higher mortality from 24h ($P < 0.01$) onwards compared to controls with only 30% of mites surviving at 72 h ($P < 0.001$) (Fig. 6b). In repeated independent experiments (not shown), the level of mortality in dsRNA-AST treated *Varroa* was consistently 50 – 60% higher than the dsRNA-LacZ treated *Varroa* over similar time spans. Though AST-knockdown increased *Varroa* mortality, no obvious phenotypic changes were observed.

The level of gene knockdown for mites treated with dsRNA-CHH was ~55% after 48h compared to the dsRNA-LacZ treated controls ($P <0.03$) (Fig. 7a). Mortality levels in *Varroa* treated with dsRNA-CHH were greater compared to dsRNA-LacZ treated mites attaining statistical significance after 72 h ($P < 0.05$). Survival after 96 h was 68% compared to 90%

in control groups (P<0.05) (Fig. 7b). In a cohort of *Varroa* treated with dsRNA-CHH a notable "shaking" or "shivering" phenotype was observed in which the mite's whole body shook from side to side while still on their feet and occurred whether the mite was on the bee larva or the Petri dish. This "shaking" was observed in all repeated experiments using different preparations of dsRNA-CHH and mites from different hives and was not observed in any mites treated with dsRNA-LacZ or dsRNA-AST. Because the *Varroa* were in groups and not individually marked, we could not determine if the "shaking" phenotype preceded death or if those mites recovered, but it was noted that groups of mites in which we observed "shaking" mites tended to have higher numbers of dead mites at the subsequent observation time point.

4. **Discussion**

Hormones and neurotransmitters play key roles in development and physiology (Kwon et al., 2001,Friis et al., 2004,Cabrera et al., 2009) and this paper presents the first transcriptome from *Varroa* and the first synganglion transcriptome in any mite. Information about neural targets, such as membrane channels, hormones and receptors provide an opportunity in the development of chemicals to control *Varroa* (Wolstenholme et al., 2007) while the RNAi gene knockdown approach described in this paper could be employed for rapid genetic validation of a given target ahead of the long chemical validation process.

Next-generation sequencing technologies are developing rapidly and are revolutionising genomics and transcriptomics but Sanger DNA sequencing techniques, such as presented here, still have an important role to play in the identification of physiological pathways that may be key to finding targets for pest control (Schwarz et al., 2013). Indeed even from the modest EST data presented here, we identified two hormones and multiple transmembrane receptors that are potential control targets, including a nicotinic acetylcholine receptor subunit, the target of neonicotinoids. The majority of contigs we sequenced mapped directly onto the first draft of the *Varroa* genome and 42.5% had putative identity to existing genes. However, this leaves 57.5% that still have no known function. Indeed 15 transcripts, or 3.7%, were not evident in the *Varroa* genome. This paucity of information highlights the limited molecular resources available to *Varroa* biologists and although the number of transcriptomic projects from closely related mites such as *Tetranychus urtica* (Bryon et al., 2013) and *M. occidentalis* (Hoy et al., 2013) is increasing, further work on annotating and characterising the *Varroa* genome and available transcriptome data is needed.

Within the *Varroa* synganglion library we identified relatively high levels (3/555 clones) of a pathogenic virus transmitted from *Varroa* to bees. DWV is almost ubiquitous in honey bee colonies and its transmission and association with *Varroa* increases significantly its pathogenicity to honey bees (Genersch and Aubert, 2010, Ryabov et al., 2014). DWV is closely related to VDV-1, with 84% nucleotide (95% amino acid) identity (Ongus et al., 2004) and this clade of viruses contribute significantly to the collapse of honey bee colonies. Sequence analysis of EST transcripts from the *Varroa* synganglion EST revealed the presence of three contigs with nearly 100% identity to *Varroa destructor* virus-1, spanning the 3' end of the viral sequence but no DWV transcripts were found. RT-PCR using virus specific primers confirmed the presence of VDV-1 in the synganglion library and revealed it was present throughout the gut, Malpighian tubules and in whole mites demonstrating no dramatic tropism for a particular tissue type. Previous studies have demonstrated the presence of three modular regions in the genomes of DWV-like viruses the 5'-UTR, the leader/capsid-encoding region and the region encoding the non-structural proteins, which can be exchanged between related viruses (Moore et al., 2011). No DWV was found in either the synganglion library by RT-PCR, which was made from 50 individuals, or in other mite tissues. This is surprising given that specific primers spanning the recombination point at the capsid / non-structural boundary detected not only the VDV-1 virus, but the recombinant DWV /VDV-1 strain. This is in agreement with the sequencing data from the transcriptome and differs from recombinants found previously (Moore et al., 2011) suggesting that virus recombination and mutation is not homogenous across *Varroa* populations. There is some disagreement as to whether DWV or VDV-1 replicates in *Varroa* (Ongus et al., 2004), (Yue and Genersch, 2005), (Shah et al., 2009) but using strand-specific qPCR we were able to demonstrate that negative VDV-1 strands were present in the synganglia, gut, Malpighian tissue and whole mites. This widespread replication could have implications in transmission of high titres of pathogenic virus to honey bee pupa.

The presence of positive and negative strand VDV-1 in the synganglia of *Varroa* could be significant in its transmission cycle. Thogoto virus in ticks has previously been shown to replicate in the tick synganglia where it is transmitted along neural tissue directly to the salivary glands for transmission to the host via salivary secretions (Kaufman et al., 2001,Kaufman and Nuttall, 1999). A similar mode of transmission of VDV-1 from *Varroa* to the honey bee may occur. Termed the "Host Manipulation Hypothesis", it is well

documented that pathogenic and parasitic organisms change the arthropod vector behaviour to enhance transmission, including for arthropod-borne viruses (Ingwell et al., 2012). For example, tick-borne encephalitis virus alters the tick vector behaviour to exhibit more aggressive and risky behaviour which favours transmission of the virus to the mammalian host (Belova et al., 2012). Replication of VDV-1 in the *Varroa* synganglion, as demonstrated in the present study, could similarly alter *Varroa* behaviour to favour transmission.

A wide range of neuropeptides from arthropods have been characterized but the majority are from the Hexapoda and Crustacea with very few from mites and ticks (Christie et al., 2011). Genetic analysis estimates that acari diverged from insects and crustacean 750 million years ago (Weinstock et al., 2006) and *Varroa* from ticks over 300 million years ago (Cornman et al., 2010) and so it does not necessarily follow that neuropeptides from acari have modes of action similar to either insects and crustacean. Most chelicerete neuropeptides characterized come from just two species of tick, *I. scapularis* (Neupert et al., 2009,Neupert et al., 2005,Simo et al., 2013,Veenstra, 2009) and *Dermacentor variabilis* (Bissinger et al., 2011). We present here a CHH-like peptide with motifs and conserved cysteines in common with previously characterized members of the CHH-superfamily that is expressed in both the synganglion and in isolated gut tissue and Malpighian tubules. It is unknown whether our preparations of gut tissue that showed presence of mite CHH-like peptide by RT-PCR were picking up transcripts from neurons innervating the gut or from gut tissue *per se*. In crustaceans, CHH-like mRNA transcripts have been reported in heart, gills, antennal gland and even the spermatophore sac (Webster et al., 2012). In insects, CHH-like mRNAs have not been found outside the central and peripheral nervous system except in *Tribolium castaneam* where transcripts are abundantly expressed in the midgut at the late larval stage (Begum et al., 2009).

Although a member of the CHH-superfamily, the *Varroa* CHH-like peptide is phylogenetically distinct from the currently characterized superfamily subgroups, falling into a clade shared with other acari and this may be reflected in its functional role. To date, no functional characterization of any acarine CHH-like neuropeptide is available (Christie et al., 2011). CHH-superfamily peptides can be split into subgroups that have distinct roles in arthropods (Jia et al., 2012). CHH1 and CHH2 primarily regulate blood glucose metabolism due to environmental conditions (Chung and Zmora, 2011,Chung et al., 2010). Members of

the MIH subfamily inhibit ecdysteroid synthesis and delay moulting in crustaceans (Nakatsuji et al., 2009). The closely related subgroup GIH, also called vitellogenesis-inhibiting hormone (VIH), suppress the production of methyl farnesoate influencing vitellogeneisis and somatic and gonadal growth (Wainwright et al., 1996). Ion Transport Protein (ITP) peptides found in locusts has shown that they regulate Cl^- ion mediated water resorption in ileum (Audsley et al., 1992) and it has been proposed that this is similar to the CHH role in osmoregulation during moulting in crabs (Chung et al., 2010), (Carley, 1978). In Tribolium beetles RNAi knockdown of ITP variants suggested a role in moulting and in egg production (Begum et al., 2009)

Clearly, the CHH-superfamily neural peptides are multifunctional and exhibit multiple physiological roles. Although phylogenetic analysis determined the L1C01 contig resides within a distinct acarine clade, no inference of function was gleaned from this analysis. The *Varroa* used for construction of the synganglion library in this study were all mature, mated, adult females emerging from brood cells. Such *Varroa* have moulted to the last stage and have fully formed ovaries and have mated, hence, the *Varroa* CHH-like is unlikely to exhibit moulting-inhibiting or gonad-inhibiting activity of MIH and GIH, respectively, in these individuals. However, these *Varroa* do search for new brood cells containing bee larvae where they will lay their eggs, but this oviposition does not take place until such brood cells have been located and certain cues trigger vitellogenesis (Frey et al., 2013). So, it is plausible that the *Varroa* CHH-like neuropeptide identified in the present study exhibits the vitellogenesis-inhibiting activity of a VIH neuropeptide. Using the RNAi gene-knockdown methods described in this paper in a reverse-genetics approach, researchers could ascertain whether the *Varroa* CHH-like neuropeptide is involved in arrested vitellogenesis and oviposition.

Allatostatins (ASTs) are a neural peptide superfamily found across a range of taxa and are pleiotropic in function including being involved in the inhibition of juvenile hormone synthesis in insects (Stay and Tobe, 2007). Three families of ASTs (A-, B-, and C-type) have been identified, to date. EST sequence analysis identified a B-type AST from *Varroa* that is expressed specifically in the synganglion and elicits a lethal phenotype when knocked-down by RNAi. In the chelicerates, ASTs have been recently identified by *in silico* analysis (Christie et al., 2011,Veenstra et al., 2012), but are yet to be functionally characterised. The B-type family of ASTs are C-terminally amidated peptides with tryptophan in the second and

562 ninth positions, and are termed the W(X)6Wamide ASTs. This family of peptides is
563 multifunctional and function as myoinhibitory peptides but also demonstrate
564 prothoracicostatic activity (Bendena and Tobe, 2012). The *Varroa* B-type AST pro-hormone
565 contains cleavage sites and W(X)6W motifs that suggest each pro-hormone is processed into
566 three distinct hormone peptides that are significantly different in sequence but each retain the
567 W(X)6W motif. Differences in sequence in small hormonal peptides can have huge effects on
568 the efficacy of hormones of different target receptors and it may be that the three predicted
569 peptides cleaved from the pro-hormone could have different functions or distribution. A
570 putative B-type AST from *M. occidentalis* has similar cleavage and peptide structure to
571 *Varroa*, but a B-type AST isolated from *T. urtica* contains six putative hormone peptides. No
572 functional characterisation of chelicerate allatostatins has yet been determined, however, the
573 highly lethal phenotype presented here on adult female *Varroa* demonstrates that it may play
574 a crucial role outside of reproductive and moult physiology. Gene-knockdown of the *Varroa*
575 AST by the non-invasive immersion approach demonstrated in the present study would
576 permit investigations into the functional role of this neuropeptide hormone. Indeed, the
577 demonstration that the expression of genes within the central nervous can be readily
578 suppressed suggests this approach could be applied more broadly to other neuropeptides and
579 in other arthropods.
580
581 dsRNAi gene knockdown was first described in *Varroa* on a mu-type glutathione S-
582 transferase (Campbell et al., 2010) demonstrating that gene expression in the adult *Varroa*,
583 can be specifically inhibited by microinjection or the direct soaking of mites with target
584 specific dsRNA. It has been shown in ticks (Karim et al., 2008) and *Drosophila* (Dzitoyeva et
585 al., 2001) that when dsRNA is injected into the hemocoel, the dsRNA can affect the exposed
586 synganglia. We have confirmed this finding in *Varroa* by targeting neuropeptides and
587 demonstrated the effect can be achieved by the less invasive method of dsRNA
588 administration immersion which is also more amenable to higher throughput. The first
589 targets for the direct management of *Varroa* were validated by the knockdown of a cocktail
590 of 14 targets that reduced *Varroa* populations in mini-honey bee colonies by 53% compared
591 to control (Garbian et al., 2012). In this current study we demonstrate the first evidence of
592 direct lethality to individual mites using a single target with dsRNA-AST killing 54% mites
593 after 72 hours compared to controls. Mortality was also seen in mites treated with dsRNA-
594 CHH with ~25% mortality compared to controls after 72h. In addition a repeatable and
595 significant cohort of dsRNA-CHH treated mites displayed a shaking phenotype after 24-48h.

Field studies of RNAi knockdown in *Varroa* targeted a range of 14 housekeeping genes (Garbian et al., 2012) rather than neural peptides, and it may be that increasing the number of neural targets, as shown here, will increase the efficacy of RNAi as a means of control.

In summary, an EST transcriptome library constructed from the synganglia of *Varroa* mites has identified pathogenic honey bee viruses, potential acaricide targets and critical neurohormones (CHH-like and B-type AST). *Varroa destructor* virus was found replicating in the synganlion and it is postulated that this may alter the behaviour of the *Varroa*. AST gene's expression was restricted to the syganglion and we demonstrated that such genes are amenable to gene-knockdown by administration of the appropriate dsRNA by an simple and trauma-free immersion method. Knockdown of the CHH and AST transcript levels demonstrates that future investigation of neurohormonal function by RNAi in mites and, more broadly, other arthropods is possible and also opens the door to the control of *Varroa* and other pest species by targeting critical genes residing in the central and peripheral nervous system.

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Figure 1. Identity and functional grouping of ESTs from synganglia into clusters. (A) BLAST analysis of transcripts classified EST clusters as mitochondrial sequences, putative identity, unknown function but present in the *Varroa* genome database or novel sequences. (B) Transcripts with a putative identity were further divided into functional categories by analysing GO identity and homology to known genes.

Figure 2. *Varroa destructor* synganglia contain pathogenic strains of deformed wing virus known as VDV-1. (A) Pairwise sequence comparison scores of synganglia EST contigs (L1A04 and L4C04) with both DWV and VDV-1. Scores represent percentage of sequence identity between pairs. (B) Detection of positive-strand virus RNA in the synganglia library was determined by an initial PCR using generic DWV primers followed by nested PCR using primers specific to four DWV recombinants. (C) Negative-strand VDV-1 virus was detected in the synganglia and gut by strand specific RT-qPCR representing replicating virus.

Figure 3. Phylogenetic tree of members of the Crustacean CHH/MIH/GIH/VIH/ITP neurohormone superfamily with homology to *Varroa destructor* L1C01. (A) Bootstrap consensus phylogenetic trees place the Acari CHH-like peptides in a separate clade from currently characterized members of the CHH superfamily. (B) Radiation phylogram of CHH superfamily places Acari CHH superfamily on a separate branch. Initial discrimination of sites by GUIDANCE (PRANK) (Penn et al., 2010) resulted in 67 conserved amino acids considered in MEGA 6 analysis (Tamura et al., 2013). Phylogeny was inferred using the Maximum likelihood method. The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the LG + G + I statistical analysis model (Jones et al., 1992) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 51 positions in the final dataset. Accession numbers are as follows; *Cancer pagurus* (CAC05347.1), *Callinectes sapidus* (AAA69029), *Scylla serrate* (AAL99355.1), *Charybdis feriata* (AAC64785.1), *Carcinus maenas* (CAA53591.1), *Cancer pagurus* **b** (2208452A), *Nephrops norvegicus* (AAK58133.2), *Macrobrachium rosenbergii* (AAL37948.1), *Penaeus japonicas* (P55847.2), *Fenneropenaeus chinensis* (AAL55258.4), *Penaeus monodon* (BAB70610.1), *Penaeus monodon* **b** (BAB69830.1), *Metapenaeus ensis* (AAC27452.1) *Trachypenaeus curvirostris* (AAL55259.1), *Eriocheir sinensis* (AAL55256.1), *Litopenaeus stylirostris* (AAL55257.1), *Procambarus clarkia* (P55848.1), *Jasus lalandii* (P83220.1), *Armadillidium vulgare* (P83627.1), *Caenorhabditis elegans* (NP_501985.1), *Homarus americanus* (gCAA38611.1), *Procambarus clarkia* **b** (BAA89003.1), *Procambarus bouvieri* (P55845.1), *Jasus lalandii* **b** (P56687.1), *Litopenaeus vannamei* (AAN86056.1), *Macrobrachium lanchesteri* (AAC36310.1), *Armadillidium vulgare* (P30814.1), *Libinia emarginata* (P56688.1), *Cancer pagurus* (P81032.1), *Carcinus maenas* **b** (P14944.2), *Penaeus japonicas* **b** (P81700.1), *Penaeus monodon* **b** (AAC84143.1), *Litopenaeus vannamei* **b** (CAA68067.1), *Marsupenaeus japonicas* **b** (BAA13481.1), *Ixodes scapularis* (XP_002400720.1), *Varroa destructor* (L1C01), *Metaseiulus occidentalis* (XP_003741125.1), *Locusta migratoria* (AAD20821.1), *Schistocerca gregaria* (AAB16823.1), *Nasonia vitripennis* (XP_001604056.1), *Tribolium castaneum* (NP_001076808.1), *Anopheles gambiae* (EAA09451.4), *Bombyx mori* (Q9NL55.1), *Manduca sexta* (AAY29658.1), *Folsomia candida* (ACF15252.1), *Drosophila melanogaster* (AAL25507.1), *Tetranychus urticae* (tetur06g00590), *Dermacentor variabilis* (ACC99599.1). CHH = Crustacean hyperglycaemic hormone; MIH = moult-inhibiting

hormone; GIH = gonad-inhibiting hormone; VIH = vitelogenesis-inhibiting hormome; ITP = ion transport peptide. *Varroa destructor* CHH shares functional and structural residues with a homologue in *M. occidentalis* (C). Methionine start codon is marked with M above sequence. Sequence highlights represent cleavage sites for Signal peptide (red with asterix above) and pro-preptide (green with arrow above). Blue highlights with C above show the location of 6 canonical cysteines intrinsic to the folding of CHH neuropeptides and yellow highlights the PFAM CHH superfamily motif.

Figure 4. *Varroa destructor* AST is homologous to B-type allatostatins in insects, crustacea and other acari. (A) Mature peptide sequences aligned in Clustal Omega all contain the allatostatin motif W(X)6W and glycine- amidinated N terminii. Conserved tryptophan residues are highlighted. Accession numbers are as follows; *M. occidentalis (XP_003741714.1), T. urtica (tetur10g00930), I. scapularis (XP_002434041.1, T castaneum (NP_001137202.1), A. aegypti (XP_001655873.1), D. ponderosae (ENN80203.1), C. quinquefasciatus (XP_001842234.1), P. humanus (XP_002422676.1), C. capitata (XP_004530893.1), M. domestica (XP_005178794.1), B. mori (P82003), G. bimaculatus (Q5QRY7), D. pulex (E9GSK4).* **(B)** Pro-hormone peptide structures vary between species with lysine / arginine cleavage sites resulting in multiple predicted mature peptides. Amino acid length is displayed after pro-peptide structure. SP = signal peptide. Numbered domains = mature peptides.

Figure 5. *Varroa destructor* CHH is distributed in both synganglion, gut and Malpighian tubule whereas AST is restricted to the synganglion. Expression of Vd-CHH and Vd-AST transcripts were determined by RT-PCR in different tissues of *V. destructor* using CHH and AST specific primers and gel loading normalized to actin. Syn = synganglion; Mite - syng = *Varroa* with synganglion removed and MT = Malpighian tubules.

Figure 6. Extent and persistence of dsRNA-VdAST gene knockdown and subsequent mortality in adult *V. destructor.* (**A**) Semi-quantitative VdAST RT-PCR band intensities were determined by densitometry and the relative abundance to the within-sample actin band calculated. The extent of VdAST : actin knockdown is presented as a percentage for VdAST-dsRNA injected mites and control mites injected with LacZ-dsRNA at each given time point. Data are means ± SEM (n = 3 individual mites). Asterisks represent significant difference ($P < 0.001$) between treatments determined by Student's t-test. (**B**) Survival was monitored post-injection with either VdAST or LacZ-dsRNA. Mites were monitored every 12h with dead mites removed from assay chambers. LacZ (4 groups of 11 mites, n=4), VdAST (3 groups of 10 mites, n =3). Data are means of each chamber ± SEM. Asterisks represent significant differences between treatments (**= $P < 0.01$; *** = $P<0.001$) determined by Student's t-test.

Figure 7. Extent and persistence of dsRNA-VdCHH gene knockdown and subsequent mortality in adult *V. destructor.* (**A**) Semi-quantitative VdCHH RT-PCR band intensities were determined by densitometry and the relative abundance to the within-sample actin band calculated. The extent of VdCHH : actin knockdown is presented as a percentage for VdCHH-dsRNA injected mites and control mites injected with LacZ-dsRNA at each given time point. Data are means ± SEM (n = 3 individual mites). Asterisks represent significant difference ($P < 0.001$) between treatments determined by Student's t-test. (**B**) Survival was monitored post-injection with either VdCHH or LacZ-dsRNA. Mites were monitored every 12h with dead mites removed from assay chambers. Injected mites assigned to groups of 4, LacZ (3 groups of 7, n = 3), VdCHH (3 groups of 7 mites, n = 3). Data are means of each chamber ± SEM. Asterisks represent significant differences between treatments ($P < 0.03$).

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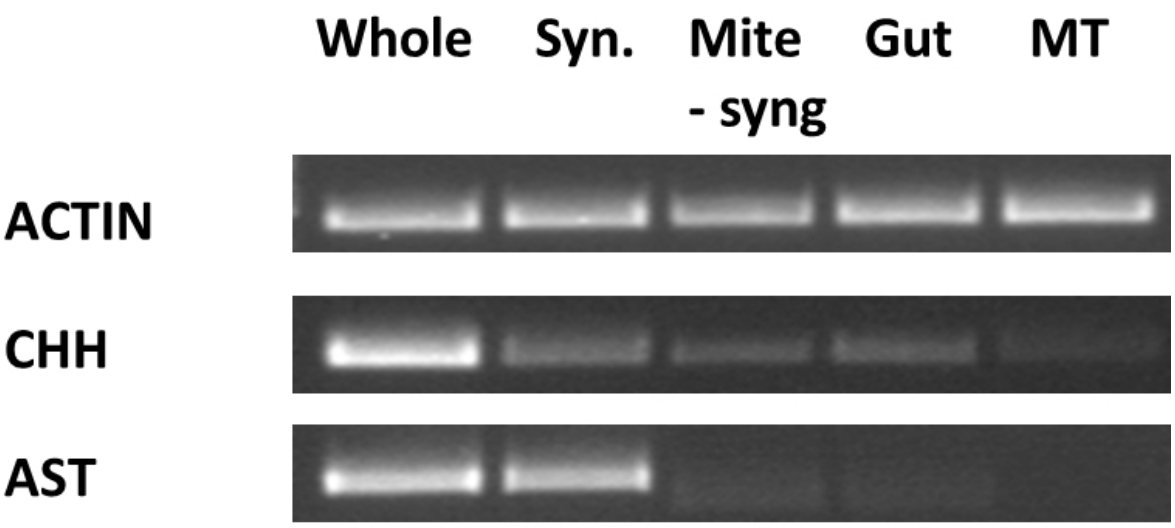
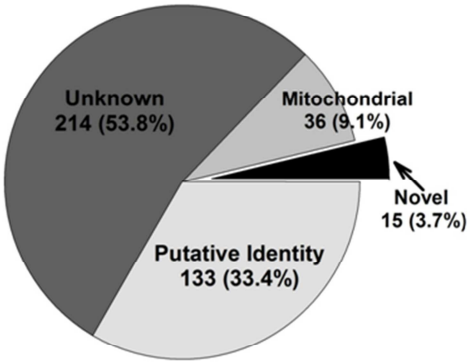


A.



B.

Functional classification	No. of clusters	%
Metabolism	26	24.7
Cell growth, division and RNA synthesis	22	20.3
Protein synthesis - translation	15	13.8
Protein association	14	12.9
Cell signaling and adhesion	7	6.4
Transmembrane receptors	5	4.6
Structural & skeletal	4	3.7
Transport	3	2.7
Hormone	2	1.8
Viral	1	0.9
Antioxidant	1	0.9
Miscellaneous	8	7.4
Total	**108**	

Figure 1. Identity and functional classification of clusters.

A.

	DWV	VDV-1
DWV	-	90.78
VDV-1	90.78	-
L1A04	90.78	99.32

	DWV	VDV-1
DWV	-	84.06
VDV-1	84.06	-
L4C04	83.93	99.34

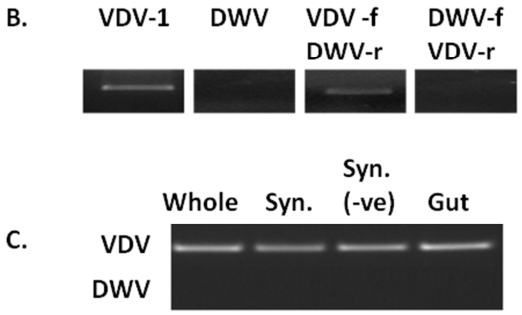
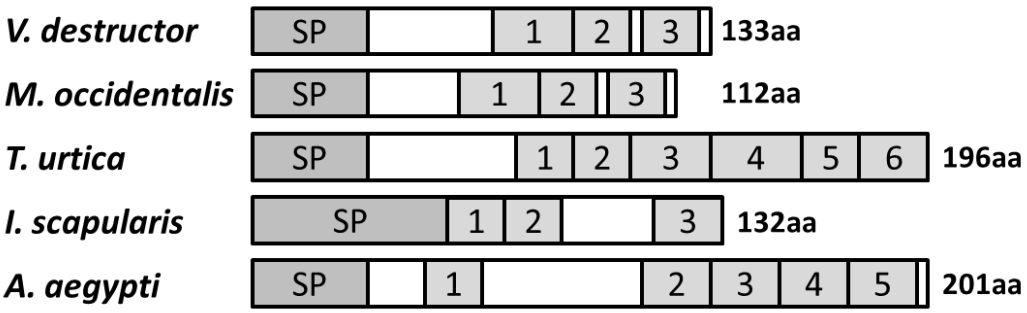


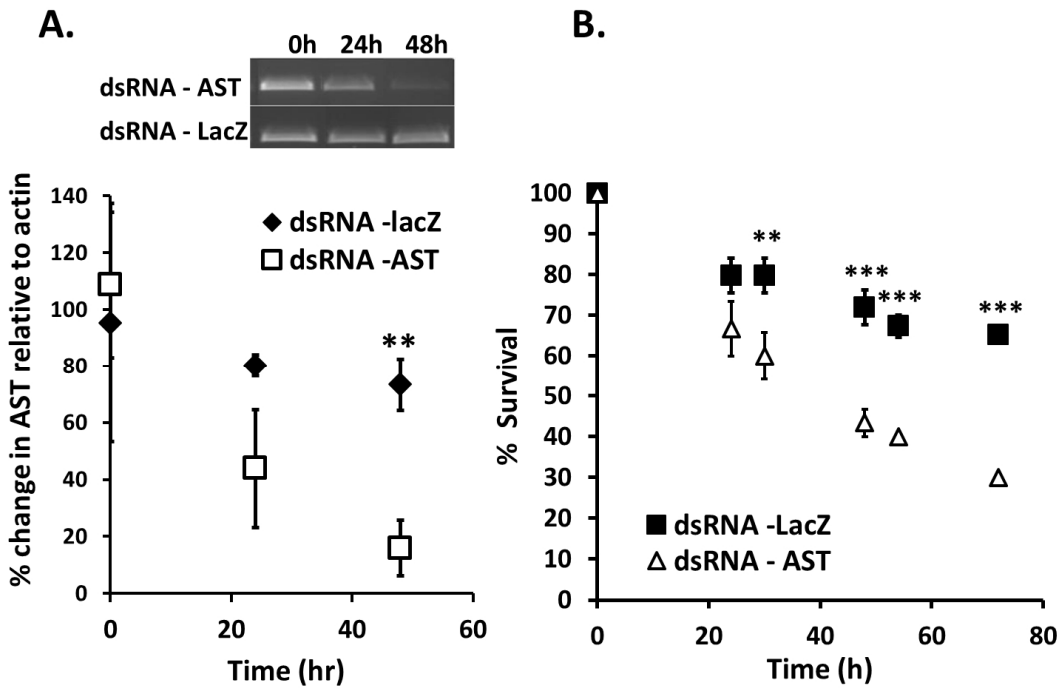
Figure 2. Varroa destructor virus was present and replicating in the synganglion of *V. destructor.*

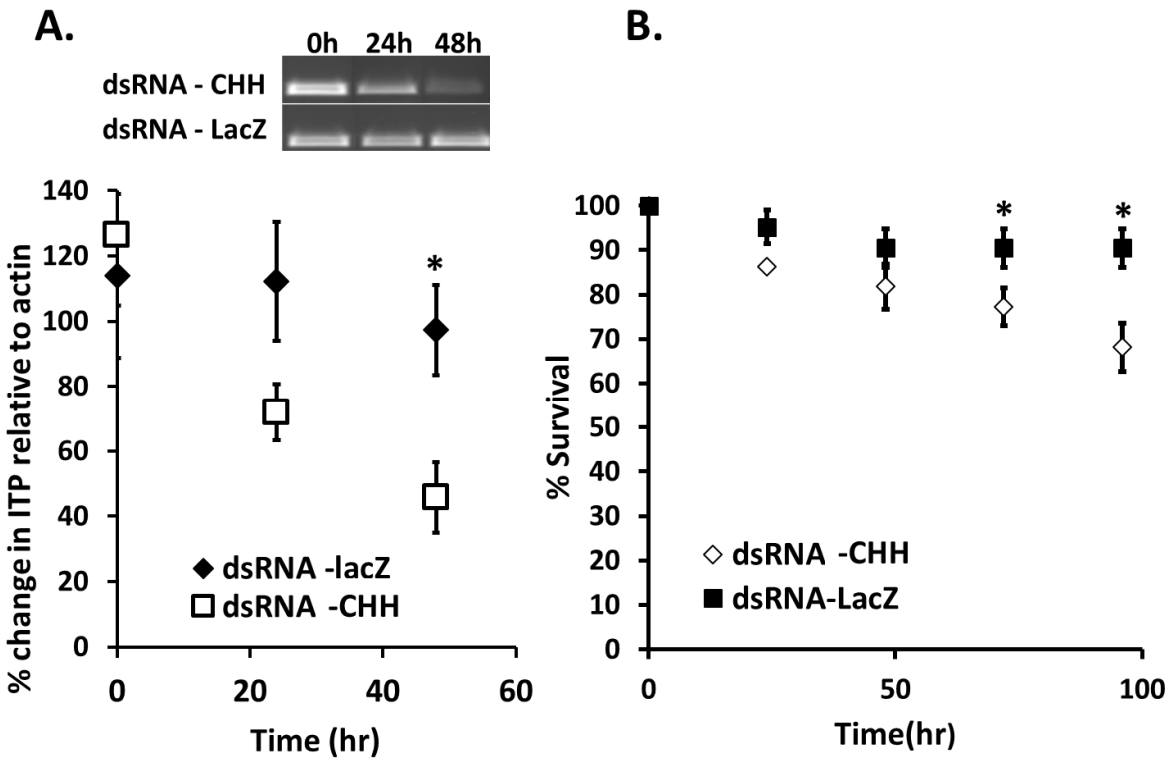
A.

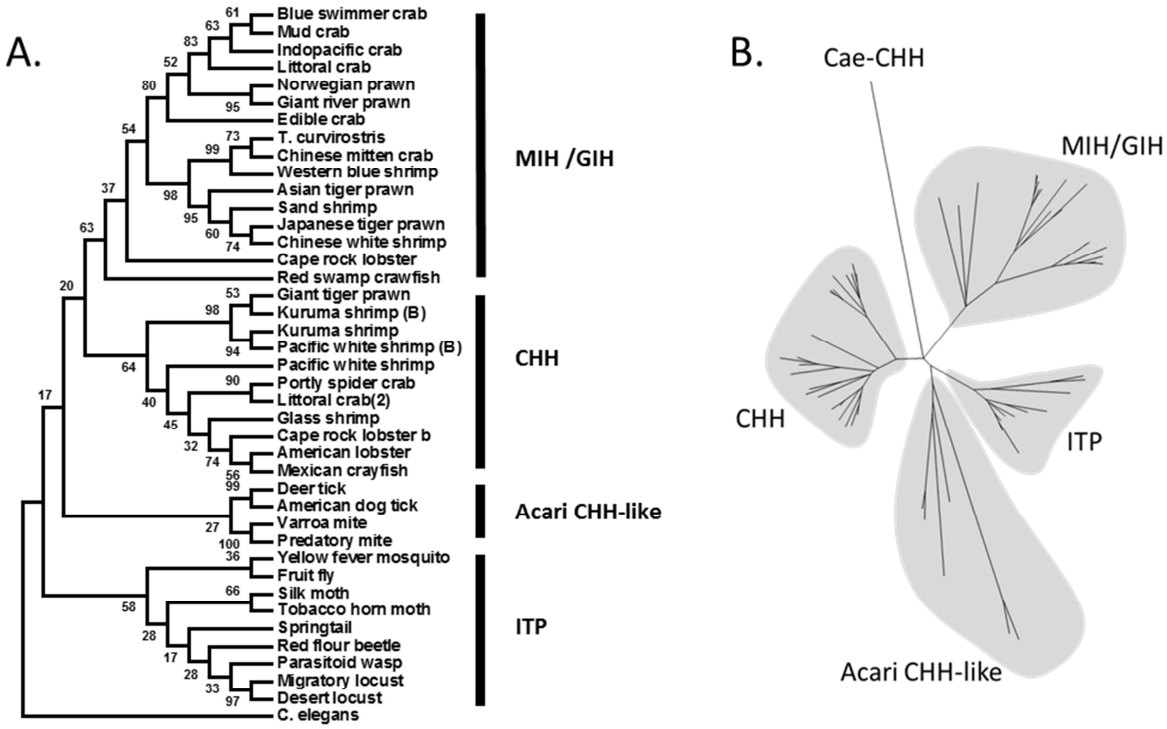
V.destructor	-PQPQ**W**NELSGY**W**G
M.occidentalis	-PQPQ**W**NELSGY**W**G
T.urtica	-DSPH**W**NNLRGM**W**G
I.scapularis	--ENH**W**NDLSGY**W**G
A.aegypti	--SEK**W**NKLSSS**W**G
D.ponderosae	--QPA**W**NNLKGL**W**G
C.quinquefasciatus	--SEK**W**NKLSSS**W**G
P.humanus	SVDSK**W**AKLQGG**W**G
C.capitata	S-SKD**W**AKLHGG**W**G
M.domesticus	---AT**W**QKLHGG**W**G
T.castaneum	---SK**W**DNFRGS**W**G
B.mori	----A**W**SALHGT**W**G
G.bimaculatus	----A**W**NNLGSA**W**G
D.pulex	----S**W**TQLHGV**W**G

B.









C.

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                                M                   *                   *     >       C
V. destructor                   MGRSYKKLSAWPLLVALVASSQLRGVRTQSLAGF---EPLGGFAGATGTMVLHKRLFLDADCRGPYAP
M. occidentalis                 MAR----------------------KDPNVVIAFAILSLLGHSC--RAFGPVHKRLFYTAECRGDYAP
                                *.*                      : : .*   . **  .   .   :*****  *:*** ***

                                      C  C                C  C        C                 <
V. destructor                   HFYGYLNRMHNICKECADMYPGMRDFISRNCTSECFRNRVFQDCVSATMQLHQLDEISNMIGQLSGRR
M. occidentalis                 HFDDYISRVDTICRECSEMYPTMRDFIAKNCSSECFRNRVFQDCMSATMQLHQLTEIHNMIGELSGRR
                                **  *:.*:..**:**::*** *****::**:************:********* ** ****:*****
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Highlights:

- The first transcriptome of *Varroa destructor*
- Deformed wing virus variant (VDV-1) present and replicating in *Varroa* synganglion ("brain")
- B-type allatostatin (*Vd*AST) and a crustacean hyperglycaemic hormone (*Vd*CHH) identified in *Varroa* synganglia
- *Vd*AST and *Vd*CCH expression knocked down by dsRNAi resulting in significant mortality